AD _____

GRANT NUMBER: DAMD17-98-1-8307

TITLE: Targeting Breast Cancer with Anti HER2/neu Diabodies

PRINCIPAL INVESTIGATOR: Louis M. Weiner, M.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR:
U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (22 Jun 98 - 21 Jun 99)

4. TITLE AND SUBTITLE
Targeting Breast Cancer with Anti HER2/neu Diabodies

5. FUNDING NUMBERS
DAMD17-98-1-8307

6. AUTHOR(S)
Louis M. Weiner, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The objective of this proposal is to develop new therapeutic reagents for breast cancer. It is our hypothesis that improved diabody-based molecules with affinity for HER2/*neu* can be engineered and will prove to be effective vehicles for the RIT of breast cancer. The first Technical Objective (T.O.) has focused on optimization of the production of the selected diabody and the identification of the optimal radionuclide and labeling strategy for diabody-based RIT. This T.O. has involved an investigation into the impact on diabody targeting and RIT of a variety of factors likely to be encountered in a clinical setting. These include the degree of antigen density, the route (i.v. bolus or continuous infusion) and frequency of administration, the presence of disseminated disease, and the effect of antigen expression on normal tissues. These experiments have set the stage for proceeding to the clinical evaluation of diabody-based targeting of breast cancer in our second Technical Objective. The clinical component of this proposal will entail a Phase I radioimmunoimaging and radioimmunoguided surgery trial designed to elicit information on the dosimetry, specificity and tumor penetration properties of radiolabeled C6.5 diabody, and will assess the RIT potential of this molecule.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

( ) Where copyrighted material is quoted, permission has been obtained to use such material.

( ) Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

( ) Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

(✓) In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

(N/A) For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

(N/A) In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Insitutes of Health.

Principal Investigator's Signature

7/20/99
Date

Table of Contents

TABLE OF CONTENTS:

INTRODUCTION:

The objective of this proposal is to develop new therapeutic reagents for breast cancer. It is our hypothesis that improved diabody-based molecules with affinity for HER2/*neu* can be engineered and will prove to be effective vehicles for the RIT of breast cancer. The first Technical Objective (T.O.) focuses on the optimization of the production of the selected diabody and the identification of the optimal radionuclide and labeling strategy for diabody-based RIT. This T.O. also involves an investigation into the impact on diabody targeting and RIT of a variety of factors likely to be encountered in a clinical setting. These include the degree of antigen density, the route (i.v. bolus or continuous infusion) and frequency of administration, the presence of disseminated disease, and the effect of antigen expression on normal tissues. Completion of these experiments will set the stage for proceeding to the clinical evaluation of diabody-based targeting of breast cancer in our second Technical Objective. The clinical component of this proposal (to be initiated in year 3) will entail a Phase I radioimmunoimaging and radioimmunoguided surgery trial designed to elicit information on the dosimetry, specificity and tumor penetration properties of radiolabeled C6.5 diabody, and will assess the RIT potential of this molecule.

BODY:

T.O.1.

Task: Determine effects of antigen density on diabody targeting (4.1.A).

Flow cytometry assays were performed to determine the impact of HER2/*neu* antigen expression on cell surface retention of the C6.5 diabody. In this assay, biotinylated C6.5 diabody was incubated with a number of cell lines expressing a range of HER2/*neu*. The expression of HER2/*neu* on the cells was first determined based upon the mean fluorescent index (MFI) resulting from the binding of a control IgG, 520C9, that is specific for HER2/*neu*. The cell lines and their MFI value (520C9/HER2/*neu*) are as follows: the OVCAR-3 human ovarian cancer line (2.9), the SK-BR-3 human breast cancer line (135.0), the SK-OV-3 human ovarian cancer line (251.2), the BT-474 human breast cancer line (322.0) and the N87 human gastric carcinoma line (430.1). Methods: one μg (20 μg/ml) of biotinylated C6.5 diabody was incubated with 4 x 10^5 target cells for 30 minutes at 4°C. All samples were washed with ice cold FACS buffer two times. Half of each sample was then fixed in 1 % paraformaldehyde. The 2nd half of each sample was resuspended in fresh FACS buffer, incubated for one hour at 37°C and then was washed and fixed as described above. Streptavidin-phycroerythrin was then added to all the tubes and the samples were incubated for 30 minutes at 4°C. The samples were again washed two times in ice cold FACS buffer and were fixed again with 1 % paraformaldehyde. The cells were assayed on a Becton Dickinson FACscan flow cytometer and the fluorescence at time zero and one hour were compared. In this assay, the retention of the diabody on the surface of the target cells was directly dependent upon the quantity of HER2/*neu* present on the cells (Table 1). In general, the cells expressing large quantity of HER2/*neu* had similar MFIs at zero minutes and one hour while the half

of the diabody had dissociated from the lowest HER2/neu expressing cell line (SK-BR-3). This supports our expectation that the diabody will bind divalently to cells expressing large quantities of target antigen, increasing the avidity and thus the duration, of association. In contrast, the diabody should predominantly bind monovalently to cells expressing low quantities of antigen, and therefore exhibit more rapid dissociation kinetics.

Table 1. Impact of HER2/*neu* expression on cell surface retention of C6.5 diabody.

	Cell line				
	OVCAR-3	SK-BR-3	SK-OV-3	BT-474	N87
[HER2][a]	2.9	135	251	322	430
% Retained at 1 hr.	0	48.2	100%	100%	87.5

[A] Relative HER2/neu expression (MFI) as determined by flow cytometry with the anti-HER2/neu antibody 520C9.

Task: Production of mice transgenic for kinase-deficient mutant of HER2/neu (4.1.D).

During the past year we have successfully expanded the colony of transgenic mice. These mice were assayed for the presence of the transgene by PCR and southern blotting and mice containing the desired gene were isolated to serve as breeders. A manuscript describing this work is currently in press in Cancer Immunology and Immunotherapy.

Task: Initiate preclinical Radioimmunotherapy (RAIT) studies (4.1.B).

Preclinical RAIT studies were performed with the C6.5 diabody using two different radiometals, Y-90 and Bi-213, as cytotoxic agents. The isotopes were selected based upon their ability to be tightly bound by the CHX-A" chelate and upon their disparate physical characteristics. Y-90 has a half-life of 2.67 days and emits a high-energy beta particle that has been proven to be a very effective agent for RAIT applications. Bi-213 has a very rapid half-life (45 minutes) and has an alpha particle as its primary emission. There were two primary goals of these trials, to determine the maximum tolerated dose (MTD) of the agents and to determine the efficacy of the treatment.

In the Bi-213 RAIT trial nude mice, bearing early (7-day old) s.c. SK-OV-3 tumors that overexpress the HER2/neu target antigen, were injected with 0.64 mCi, 0.4 mCi or 0.15 mCi of Bi-213-CHX-A" C6.5 diabody or unlabeled C6.5 diabody. The mice were observed for signs of toxicity (weight loss) and tumor growth. All three radioactive dose levels of Bi-213-CHX-A" C6.5 diabody were associated with major toxicity and little therapeutic efficacy. From this study we concluded that the half-life of Bi-213 is too rapid to allow sufficient localization of the labeled diabody in the tumor for a therapeutic effect. As a result, we initiated therapy studies using the longer half-life isotope Y-90 on the C6.5 diabody, described below.

The Y-90-CHX-A" C6.5 diabody study was performed in nude mice bearing established 30-day old s.c. SK-OV-3 tumors. Groups of mice were given 0.5, 0.3, 0.2, 0.1, 0.05 mCi of Y-90-CHX-A" C6.5 diabody or unlabeled C6.5 diabody by i.v.injection and the mice were observed for signs of toxicity and therapeutic efficacy as above. In contrast to RAIT with the Bi-213 conjugate described above, the MTD for the Y-90 conjugate occurred between the 0.3 and 0.5 mCi doses. At the 0.3 mCi dose, 5 of the 6 mice survived the treatment and each experienced little or no weight loss. However, with the 0.5 mCi dose all of the mice experienced weight loss (7-30 %) and 5 of the 7 treated animals died. In terms of efficacy, by day 25 post treatment, the mean tumor volumes of the 0.3 and 0.5 mCi dose group mice were 2 and 4 - fold, respectively less than that of the control mice.

As the SK-OV-3 tumor cell line employed in these studies has a mutated form of P53, it is incapable of undergoing apoptosis is response to exposure to high levels of ionizing radiation. Accordingly, we are currently preparing to reevaluate RAIT using the Y-90-CHX-A" C6.5 diabody in mice bearing MDA-MB 361/DYTR tumors that overexpress HER2/*neu*. This cell line was recently acquired from Dr. Da Jun Yang of Georgetown University as it has proven to be an effective target in RAIT studies.

Task: Perform in vitro characterization of affinity mutant diabody molecules.

In the interval between the submission of the grant application and the notification of funding, we constructed two additional diabodies from affinity mutants of the C6.5 scFv. During the past year we have evaluated the *in vitro* properties of these molecules. A comparison between the binding affinities of the scFv and diabody forms of each of the molecules is presented in Table 2. In general, the increase in affinity resulting from the diabody format appears to be greater when the parent scFv is of a lower affinity. Both C6G98A (1.3×10^{-7} M) and C6.5 (2.5×10^{-8} M) demonstrated approximately a 15-fold increase in affinity as a result of expression in a diabody format. In contrast, the affinity of C6ML3-9 (1.0×10^{-9} M) only increased about 3-fold as a result of being expressed in a diabody format.

Table 2. Binding properties of C6.5 affinity mutant scFv and diabody molecules. Values were determined by surface plasmon resonance on a BIAcore instrument.

Clone	scFv/diabody	k_{on} (10^5 s^{-1} M^{-1})	k_{off} (10^{-3} s^{-1})	K_d (10^{-9} M)
C6G98A	scFv	4.1	55	133
	diabody	4.6	3.7	8.0
C6.5	scFv	4.0	10	25
	diabody	5.2	0.84	1.6
C6ML3-9	scFv	7.6	0.76	1.0
	diabody	7.7	0.28	0.36

Task: Begin preliminary in vivo characterization of diabody molecules with favorable characteristics.

In vivo biodistribution and cumulative organ retention or area under the curve (AUC) studies were performed in scid mice bearing established SK-OV-3 tumors. The diabody molecules described above were trace labeled with I-125 and their quality was confirmed in immunoreactivity assays prior to initiating the biodistribution studies. Twenty micrograms of diabody were administered by i.v. injection and cohorts of five mice were euthanized at multiple time points (e.g., 1, 4, 24, 48, and 72 hrs post injection). The retention of the radiolabeled diabody in each organ was determined as described in the methods section of the grant and the cumulative retention (AUC) was determined using the NCOMP program. In these studies, the tumor retention of the two lower affinity diabodies (C6G98A and C6.5) were clearly superior to that of the highest affinity diabody (C6ML3-9) (Table 3). Furthermore, the tumor: blood AUC ratio and corresponding tumor:marrow ratio was observed with the original C6.5 diabody. As a result of these studies, we have decided to focus our future efforts on the development of the C6.5 diabody for the upcoming phase I clinical trial (year 3).

Table 3. Tumor:Blood and Tumor:Marrow AUC values for the affinity mutant diabody molecules. Values were determined by performing biodistribution experiments with radioiodinated molecules in SK-OV-3 tumor bearing *scid* mice. Area under the curve (AUC) values describing cumulative organ residence were determined using the NCOMP program described in the grant proposal. Marrow values were estimated to be 25% of the corresponding blood values.

Clone	Tumor:Blood AUC	Tumor:Marrow AUC	%ID/g Tumor at 24 Hr.
scFv			
C6G98A	n.d.	n.d.	0.19
C6.5	2.4:1	9.6:1	1.32
C6ML3-9	n.d.	n.d.	1.42
diabody			
C6G98A	2.4:1	9.6:1	7.07
C6.5	3.0:1	12.0:1	6.48
C6ML3-9	1.8:1	7.2:1	3.18

KEY RESEARCH ACCOMPLISHMENTS (YEAR 1):

- Determined that a positive correlation does exist between HER2/*neu* antigen density and C6.5 diabody retention of the cell surface.
- Identified transgenic mice that contain the HER2/*neu* gene (by PCR and southern blot) to serve as breeders for expansion of colony.
- Initiated preclinical RAIT studies with therapeutic radiometal conjugates.
  - Determined MTDs for Bi-213 and Y-90 conjugates.
  - Determined that Bi-213 half-life is too rapid for use with the diabody.

- Observed partial responses in SK-OV-3 tumor-bearing mice to treatment with Y-90 conjugated diabody.
- Created diabodies from two C6.5 affinity mutant scFv molecules
- Determined *in vitro* binding properties of the affinity mutant diabody molecules.
- Performed biodistribution studies with affinity mutant diabodies and concluded that the original C6.5 diabody molecule has best properties to serve as a vehicle for RAIT and RIGS.

REPORTABLE OUTCOMES:

- The work with transgenic mice is now in press (Cancer Immunologie, Immunotherapie)

CONCLUSIONS:

- Diabody-based RIT offers promise in the therapy of breast cancer
- Clinical development of the C6.5 diabody will require that a sponsor be found to make GMP-quality material suitable for human clinical trials of diabody-based radioimmunotargeting

REFERENCES:

None